1632 IFW

Practitioner's Docket No. 1822/117 ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Nissim Benvenisty

Application No.: 09/995,452
Filed: 11/27/2001
For: Transfection of Human Embryonic Stem Cells

Group No.: 1632
Examiner: Ton, Thaian N.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)

I hereby certify that the attached correspondence comprising:

Declaration of Benvenisty, M.D., Ph.D. in Support of Applicant's Response of September 20, 2004

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Signature of person mailing paper

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benvenisty	Atty Docket:	1822/117
Serial No.:	09/995,452	Art Unit:	1632
Date Filed:	November 27, 2001	Examiner:	Ton, Thaian N.
Invention:	Transfection of Human Embryonic Stem cells		

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CERTIFICATE OF MAILING

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Barbara J. Carter, Ph.D.

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF BENVENISTY, M.D., PH.D. IN SUPPORT OF APPLICANT'S RESPONSE OF SEPTEMBER 20, 2004 [37 C.F.R. § 1.132]

Dear Sir:

In support of the Response and RCE filed on September 20, 2004 submitted in reply to the Office Action of March 19, 2004 ("Office Action") and advisory action of July 9, 2004 for the above-referenced matter, I hereby declare as follows:

1. My name is Nissim Benvenisty, M.D., Ph.D. I am a Professor at The Hebrew University in Jerusalem, Israel, in the Department of Genetics. I was formerly the Vice Chair of the Institute of Life Sciences at The Hebrew University, and have been a visiting Professor in the Department of Genetics at Harvard University in Boston, MA, among other positions. I have been awarded numerous prizes and fellowships,

including the Teva Prize for excellent research in stem cells in 2003, the Herbert Cohn Chair in Cancer Research during 1999, the Hestrin Prize in Molecular Biology in 1997, a Howard Hughes Postdoctoral Fellowship from 1991-1993, and a Fulbright Postdoctoral Fellowship from 1990-1991. I have published extensively in the field of stem cell research, and other areas, with over 65 publications, to date. I am also an inventor or co-inventor of a substantial number of patents involving (among other things) human embryonic stem cell research, and I am a co-inventor of the invention claimed in the current application. My further credentials are set forth in my Curriculum Vitae, which is attached hereto as Exhibit A.

2. I have read the action of July 9, 2004. This declaration is provided to clarify the record concerning transfection of human embryonic stem cells relative to transfection of mouse and other animal embryonic stem cells. In particular, I intend a) to provide clear and convincing evidence why having the ability to transfect mouse and other animal ES cells successfully does not translate into the ability to transfect human ES cells successfully; b) to explain why it would not have been obvious to make the proposed combinations and modifications set forth in the Office Action; and c) why those skilled in the art would not expect that such combinations and modifications of mouse and other animal cell transfection protocols would be successful in achieving efficient transfection of human embryonic stem cells.

Consideration of the Prior Art

3. The Examiner asserts that the present invention is anticipated by Smith et al. because, among other reasons, "the claims do not require a particular number, concentration of percentage of cells, that are transfected by the polynucleotide of

interest, or a particular transfection efficiency." See Office action, p. 6. Claims 1 and 11 have now been amended, requiring introduction of the DNA in a transfection preparation, which has transfection reagents selected from cationic non-lipid polymer reagents, non-liposomal reagents, and cationic lipid agents, and no other, meaning that electroporation cannot be used to introduce the DNA, and adenovirus cannot be present in the transfection preparation. In light of these claim amendments, Smith et al. does not anticipate our invention.

4. The Examiner has also cited a number of references relating to methods for introducing polynucleotides into animal cell lines, including human cell lines, using various means, including electroporation, transfection in the presence of adenovirus, lipofection, and more to support rejections of all the claims for reasons of obviousness. The Examiner asserts that the motivation to modify and combine the references, set forth in the Office Action in various combinations to support the obviousness rejections, would be found by one of ordinary skill in the art, "especially in the absence of sufficient, clear and convincing evidence to the contrary." For every combination and modification, the Examiner cites specific passages and phrases from the references in support of the asserted rejection. See Office Action, p.9 for Smith with Myers; p. 11 for Smith with Fasbender; p. 13 for Smith with Fasbender with Pascolo; p. 15 for Smith with Gibco BRL catalog; and p. 16 for Thomson with Bradley.

5. I disagree with the idea that combination or modification of the cited references by researchers working in the field of hES cell research would yield successful transfection of hES cells, or that these cited references render our invention obvious.

First, it was known to those of us working in the field and trying to successfully transfect human ES cells at the time of this invention that electroporation did not introduce DNA into hES at rates or levels high enough to do meaningful research with genetically altered human ES stem cells. It is clear from our description and especially Figure 1 that transfection with chemical transfection reagents such as ExGEN™ 500, a polycationic non-lipid polymer, successfully introduced DNA into hES at levels over an order of magnitude better than other chemical reagents or electroporation.

6. Second, although Figure 1 of our application shows *relative* transfection efficiencies between the various methods examined, a quantitative value for the level of transfection achieved in hES cells using cationic polymers (transfection rates of ~10^{-5}) is included in the 2001 publication that provided much of the key data this application was based on, including Figure 1 itself (see Eiges et al., (*Curr. Biol.* **11**, 514-518 (2001), p. 515, col. 2, first para. – attached as exhibit B). This is substantially better than the transfection rates of only ~10^{-7} achieved when electroporation protocols, worked out for introducing DNA into mouse and other animal ES cells, were used on hES cells, even as late as 2003 (see Zwaka et al. (*Nature Biotech* (2003), vol. **21**, pp. 319-321 - attached as Exhibit C)).

7. More importantly, the rejections in the Office Action are based on a fundamental assumption that translating mouse and other animal transfection protocols, most developed for transfection of cell lines other than embryonic stem cells, can be easily adapted to achieve successful and efficient transfection of *human* embryonic stem

cells. This assumption is not supported by the experiences of those skilled in the art trying to solve this very problem at the time this invention was submitted.

8. According to Zwaka et al., "For human ES cells, the best chemical reagents yield stable ... transfectants at rates of about 10^{-5}; mouse ES cell electroporation procedures yield substantially lower rates.[6]" (see p. 319, col. 1, 2nd para.). The reference cited (reference 6) is Eiges et al. (see above) a reference for which I am the corresponding author, and the paper which contains much of the core experimental data that forms the basis for this invention. The 2001 Eiges et al. publication, based on research carried out in my laboratory in 2000 and before, is the first publication reporting the transfection of human ES cells.

9. Although the Examiner points out that our application states that "human ES cells can be transfected by electroporation ..." these were our own observations, not those reported by others working in the field. However, it should be emphasized that the yield obtained then (at the time of the present invention) when transfecting hES cells through electroporation was not feasible for performing further manipulations with the transfected cells and thus, we abandoned this technology and searched for a substitute. Except for other publications from my laboratory, no other researcher reported successful transfection of human ES cells under the same conditions described in the present application, until the Zwaka et al. reference in 2003, a full three years after the priority date of this application, in spite of the wealth of information available concerning how to achieve successful transfection of ES cells in other animals, or how to achieve transfection of other cell types in both humans and other animals.

10. In addition, Zwaka et al. reported in their article that when a typical mouse ES cell electroporation protocol was tried as a means for introducing DNA into human ES cells, the results were so poor, and the rate of introduction so low ($\sim 10^{-7}$), that it was not practical to use mouse electroporation protocols for introducing DNA into human ES cells (*id.*). And significantly, Zwaka et al. also state that their "... failure to achieve homologous recombination with chemical transfection reagents led [them] to re-evaluate electroporation procedures for human ES cells" (*see id.*, p. 319, first full para.), but this is a 2003 reference, indicating that until then, transfection of human ES cells using electroporation was not a viable means for introducing DNA into hES cells, and thus Smith *et al.* cannot be cited as prior art for an obviousness rejection.

11. Eiges et al. and Zwaka et al. provide substantial evidence that mouse protocols for introducing DNA into human ES cells did not work at the time this invention was submitted, even as late as 2003. The Zwaka and Eiges publications directly contradict Smith et al.'s claim, in the absence of *any* experimental evidence with human ES cells using electroporation or other means, that essentially *any* means will work for introducing a marker into *any* animal ES cells, including "transfection, lipofection, injection, ballistic missile, viral vector, or [...] electroporation" (see Smith et al., Col. 2, lines 63-64).

12. Thus, Smith et al. is only suitable for showing electroporation of *mouse* ES cells and not chemical transfection of *human* ES cells. To assume that Smith et al. or anyone else had achieved transfection of hES in the presence of cationic polymers before our invention is simply not supported by the reports of those working in the field of hES cell research at the time of our invention. Smith et al. were only capable of

transfecting *mouse* ES cells using electroporation as the means of introducing the nucleic acid selectable marker. The Smith reference does not provide examples and protocols for introducing a selectable marker into ES cells using any means other than electroporation, let alone protocols for introducing markers into *human* ES cells (see Smith et al., Examples 1-5 in Col. 6-12).

13. The cited art provides no protocols for DNA transfection into embryonic stem cells of any species other than mice. Smith et al. may state that some of its techniques are applicable to humans, but all the data provided by the examples are limited to mouse cells. Myers, Fasbender et al., and Pascolo et al. provide elements of genes that may be transferred or transfection techniques, but they provide no insight as to the success of their teachings as applied to human embryonic stem cells.

14. The lack of any previous protocol for DNA transfection into human embryonic stem cells is significant because it underscores that murine ES cell techniques cannot be assumed to work with human ES cells. As discussed earlier, electroporation may be a successful transfection technique for murine ES cells, but not for human ES cells. Also, homologous recombination techniques as taught by Bradley et al. may have some success with murine ES cells, but as Zwaka et al. showed, such homologous recombination techniques as applied to human ES cells are unsuccessful.

15. Most importantly, substantial differences exist between mouse and human embryonic stem cells. As discussed by Kaufman et al. (*Proc. Natl. Acad. Sci. USA*, Vol. **98**, No. 19, pp. 10716 – 10721, (2001) – attached as Exhibit D). ., "mouse and human ES cells differ in morphology, population doubling time, and growth factor requirements." For example, mouse ES cells can be maintained in an

undifferentiated state in the absence of feeder cells if the cell media includes leukemia inhibitory factor (LIF). In contrast, hES cells will either differentiate or die if exposed to LIF without feeder cells (*id.*). A summary of some of the key differences between mouse and human ES cells is shown in the table below, as further evidence that it was not trivial to transfect hES cells using mouse protocols:

	Human	**Mouse**
Dependency on LIF	No	Yes
Potential to differentiate into trophoectoderm	Yes	No
Surface markers	SSEA-4 positive SSEA-1 negative	SSEA-4 negative SSEA-1 positive

16. Furthermore, human and mouse ES cells also differ in their morphology and growth rate.As a result of these and other differences between mouse and human ES cells, as mentioned before, we were not able to use electroporation to transfect hES cells, so we started looking for a different technology that would enable transfection, which ultimately led us to the claimed invention. The fact that we had to look for alternatives to electroporation, in 2001, and that Zwaka et al. reiterated that electroporation protocol as performed for mouse ES cells did not work for introducing DNA into hES cells, is more evidence that the transferring of technology from one species to the other was not trivial.

17. All these differences show that one skilled in the art cannot expect that techniques that succeed in producing a particular result with murine ES cells will produce a similar result in human ES cells. Indeed, the specific failures of using a simple electroporation protocol in human ES cells indicate why it was not reasonable to expect that using murine transfection techniques would be successful in human ES cells.

18. As stated above, until our laboratory published the successful transfection of human embryonic stem cells in 2001 (see Eiges et al), no other laboratory had been able to do this. If it was as obvious as the Examiner contends to combine known transfection protocols developed for mice and other animals to achieve successful transfection of human ES cells, then why could no other laboratory do it until we did, especially considering how important the ability to efficiently transfect human ES cells is and considering how many other laboratories were trying to do just that? As stated above, even electroporation techniques were not reported as a means for introducing DNA into *human* ES cells until the techniques reported by Zwaka et al. in 2003, in spite of the unsubstantiated assertions in Smith to the contrary.

19. The work in our laboratory, as published in Eiges et al. (see exhibit B) shows that we were able to achieve transfection rates an order of magnitude better than achieved using electroporation, LipofectAMINE Plus™ or FuGENE™ when the chemical transfection reagent ExGen 500™, a polycationic non-lipid polymer reagent was used (see Eiges, Figure 1) and that the quantitative transfection rate we achieved was ~10^{-5} (see above). Bradley teaches transfection of human ES using electroporation. But again, this is an unsubstantiated assertion. As indicated above in Zwaka et al.,

the levels of successful introduction of DNA into hES cells using electroporation, even in 2003, were only ~10^{-7} and thus "too low to be practical." (see Zwaka et al., p. 319, col. 2, first para.). And until our publication in 2001 (see Eiges et al.) there is a not one published report showing successful introduction of DNA into human ES cells supported by experimental data. In contrast, we showed in our laboratory that our transfection methods using cationic polymers yielded transfection rates more than an order of magnitude better than electroporation in any system, and that "stable clones were derived in an efficiency of ~10^{-5} from the transfected cells" (see Eiges, p. 515, col. 2, last para. and as quoted in Zwaka et al., col. 1, second para.).

20. In summary, even if the combinations proposed by Examiner in the 103 obviousness rejections were made, one simply would not arrive at the subject matter claimed in the present application. The Examiner has cited a multitude of combinations to attempt to establish a *prima facie* case of obviousness against the presently claimed invention, but the combinations do not hold up. Researchers in the field of human ES cell research may have tried to combine and/or modify the teachings in the prior art of stem cell research – a hugely unpredictable field – to solve the problem of translating mice and monkey transfection and electroporation protocols to human ES cells efficiently to develop human ES cell lines with altered gene expression. However, no one was successful until my laboratory showed the way, specifically because it was *not* obvious to achieve our results based on what was already known about transfecting mouse ES cells and other animal cells.

21. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these

21. I hereby declare that all statements made herein are of my own knowledge and that all statements made on information and belief are true; and further that these statements are being made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Benvenisty, M.D., Ph.D.

Dated: October 14, 2004

01822/00117 340694.1

Exhibits:

A - *Curriculum Vitae* of Nissim Benvenisty, M.D., Ph.D.

B - Eiges et al. (2001) *Curr. Biol.* **11**, 514-518.

C - Zwaka et al. (2003) *Nature Biotech*, **21**, 319-321.

D. - Kaufman et al. (2001),*Proc. Natl. Acad. Sci. USA*, **98** (19), 10716 – 10721.

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Exhibit A

NISSIM BENVENISTY

CURRICULUM VITAE

Date and Place of Birth : 9.10.1958; Israel.

Marital Status: Married + 3.

Education :

1983	M.D.	Faculty of Medicine, Hebrew University.
1986	Ph.D.	Department of Developmental Biochemistry, Hadassah Medical School, Hebrew University.

Employment and Related Training :

2002-	Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
2002-2003	Head of Biology Teaching and Vice Chair, Institute of Life Sciences The Hebrew University, Jerusalem, Israel
1999-2000	Visiting Professor, Department of Genetics, Harvard University, Boston, USA
1998-2002	Associate Professor, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1993-1998	Senior Lecturer, Department of Genetics, The Hebrew University, Jerusalem, Israel.
1990-1993	Research Fellow, Department of Genetics, Harvard Medical School, Boston, USA. Under supervision of Professor Philip Leder.
1986-1990	Israeli Army Medical Service.
1983-1986	Graduate Student, Department of Developmental Biochemistry, The Hebrew University, Jerusalem, Israel.
1985	Research Associate, Case Western Reserve University, Cleveland, USA.

1983-1984	Internship, Hadassah Hospital, Jerusalem, Israel.
1982-1985	Teaching biochemistry and molecular biology to medical students at The Hebrew University.

Awards and Fellowships :

1981	Awarded the Faculty Prize.
1982	Awarded a Fellowship at the Mount Sinai Hospital, New York - Program for outstanding students.
1982-1985	Foulkes Foundation Fellowship.
1985	Best Teacher Award for teaching biochemistry and molecular biology.
1988	Awarded the Senta Foulkes Prize (London).
1990-1991	Awarded the Weizmann Postdoctoral Fellowship.
1990-1991	Awarded the Fulbright Postdoctoral Fellowship.
1991-1993	Awarded the Howard Hughes Postdoctoral Fellowship.
1993-1996	Awarded the Alon Fellowship.
1994	The Joseph H. and Belle R. Braun Senior Lectureship in Life Sciences.
1995-1998	Awarded Best Teacher in Genetics.
1995	Awarded the Hebrew University Prize for Young Scientist.
1996	Awarded the Naftali Prize.
1997	Awarded the Hestrin Prize in Biochemistry and Molecular Biology.
1998	Awarded the Rom Prize in Genetics
1999	The Herbert Cohn Chair in Cancer Research
1999-2000	Awarded the Yamagiwa-Yoshida Memorial International Cancer Study Fellowship.
2003	Awarded the Teva Prize for excellent research in stem cells

List of publications

1. **Benvenisty, N.**, Ben-Simchon, E., Cohen, H. Mencher, D., Meyuhas, O. and Reshef, L. : Control of the activity of phosphoenolpyruvate carboxykinase and the level of its mRNA in livers of newborn rats. Eur. J. Biochem. 132: 663-668 (1983).

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4. **Benvenisty, N.**, Mencher, D., Meyuhas, O., Razin, A. and Reshef, L. : Sequential changes in the methylation patterns of the rat phospho-enolpyruvate carboxykinase gene during development. Proc. Natl. Acad.Sci. USA 82: 267-271 (1985).

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9. **Benvenisty, N.** and Reshef, L. : Direct introduction of genes into rats and the expression of the genes. Proc. Natl. Acad. Sci. USA 83: 9551-9555
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Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells

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Human embryonic stem (ES) cells are pluripotent cell lines that have been derived from the inner cell mass (ICM) of blastocyst stage embryos [1–3]. They are characterized by their ability to be propagated indefinitely in culture as undifferentiated cells with a normal karyotype and can be induced to differentiate in vitro into various cell types [1, 2, 4–6]. Thus, human ES cells promise to serve as an unlimited cell source for transplantation. However, these unique cell lines tend to spontaneously differentiate in culture and therefore are difficult to maintain. Furthermore, colonies may contain several cell types and may be composed of cells other than pluripotent cells [1, 2, 6]. In order to overcome these difficulties and establish lines of cells with an undifferentiated phenotype, we have introduced a reporter gene that is regulated by a promoter of an ES cell-enriched gene into the cells. For the introduction of DNA into human ES cells, we have established a specific transfection protocol that is different from the one used for murine ES cells. Human ES cells were transfected with enhanced green fluorescence protein (EGFP), under the control of murine *Rex1* promoter. The transfected cells show high levels of GFP expression when in an undifferentiated state. As the cells differentiate, this expression is dramatically reduced in monolayer cultures as well as in the primitive endoderm of early stage (simple) embryoid bodies (EBs) and in mature EBs. The undifferentiated cells expressing GFP can be analyzed and sorted by using a Fluorescence Activated Cell Sorter (FACS). Thus, we have established lines of human ES cells in which only undifferentiated cells are fluorescent, and these cells can be followed and selected for in culture. We also propose that the pluripotent nature of the culture is made evident by the ability of the homogeneous cell population to form EBs. The ability to efficiently transfect human ES cells will provide the means to study and manipulate these cells for the purpose of basic and applied research.

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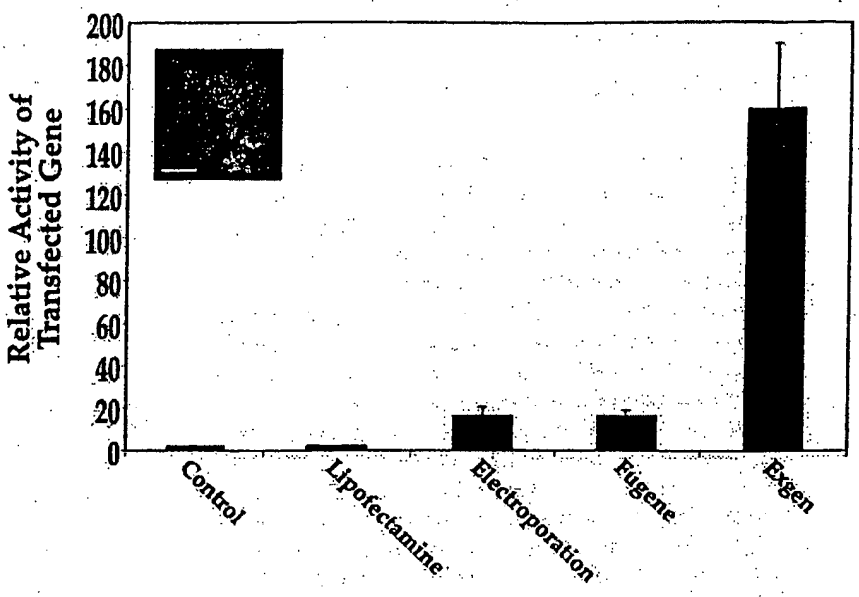
Results and discussion

The objective of this study was to obtain pure clones of human ES cells that are genetically modified so that their undifferentiated phenotype can be followed and selected for in vitro. Thus, we aimed at introducing the EGFP reporter gene under the control of a promoter of an ES cell-enriched gene into human ES cells. By tagging the undifferentiated cells with GFP, we wished to monitor the differentiation status of the cells in culture during growth and propagation as well as following spontaneous and induced differentiation. For this purpose, we chose to use the well-characterized promoter sequence of the murine *Rex1* gene [7]. *Rex1* is a retinoic acid-regulated zinc finger protein that is expressed in preimplantation mouse embryos (including the inner cell mass), trophoblast, and spermatocytes as well as in undifferentiated murine ES cells and some embryonic carcinoma (EC) cell lines [7, 8]. This gene is rapidly downregulated upon differentiation of the embryonic cells. Hence, by introducing *Rex1*-regulated gene markers (*Rex1*-EGFP) into human ES cells, we should be able to express these markers in pluripotent cells, allowing the determination of the differentiation status of these cells in culture.

In order to introduce *Rex1*-EGFP fusion gene into human ES cells, we had to establish a method to transfect the human embryonic cells with DNA. Although ES-like cell lines are now available from a large array of mammalian species (for a review, see Prelle et al. 1999 [9]), there are no published protocols for DNA transfection in any of the species, other than mice. In the mouse, electropora-

Figure 1

Transfection of DNA into human ES cells. DNA was introduced into human ES cells either by electroporation or by using several commercial reagents such as LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas). To determine the efficiency of DNA introduction by each of the methods, the cells were transfected with a construct of firefly Rennila protein under the control of a TK promoter. The cells were harvested 48 hr after transfection, and luminosity of the Rennila protein was monitored using a luminometer. Results are given in the histogram as the relative activity of the transfected gene (luminosity units per mg of total protein), following the subtraction of the values obtained from samples of the appropriate MEF-only controls. Each experiment was repeated three times, and the mean with standard error is shown. Inset: human ES cells transiently transfected with EGFP under the control of the housekeeping gene E1F (elongation factor I) promoter. Note the green fluorescent ES cells that incorporated the foreign DNA. The scale bar indicates 100 μm.

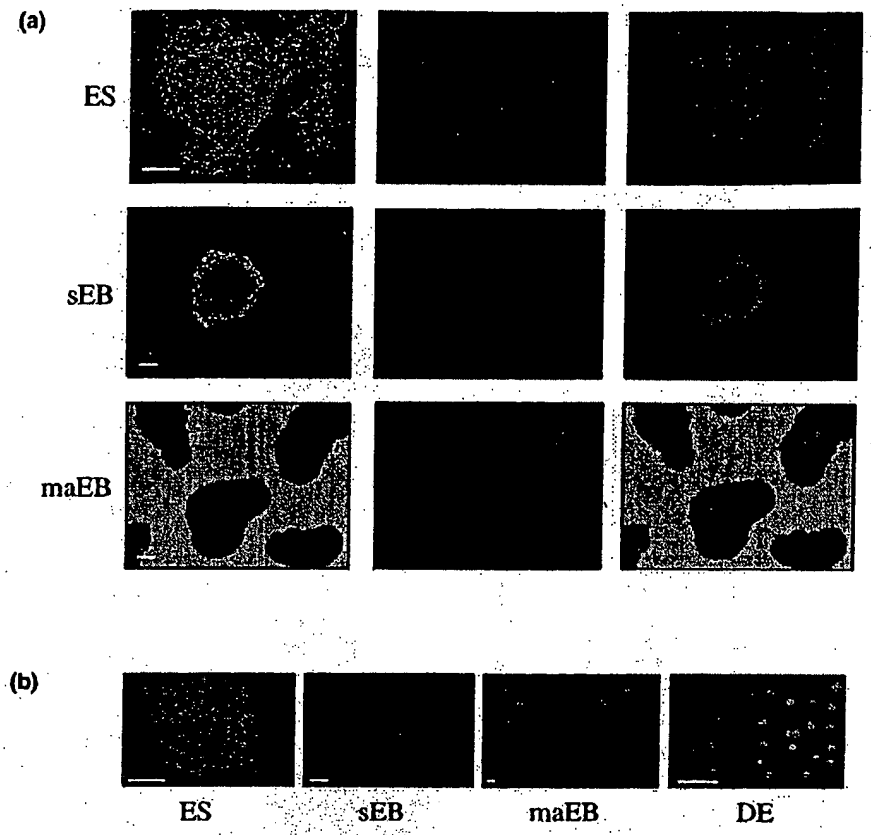


tion was found to be the method of choice for introducing foreign DNA into ES cells [10]. However, human ES cells do not survive electroporation well. Therefore, we compared the efficiencies of several chemical-based methods for the transfection of H9 human ES cells [1] (passage 40–50). Initially, an expression construct of EGFP under the control of the housekeeping gene elongation factor I (E1F) was introduced into human ES cells by several different reagents. Transient expression of the GFP was observed in no more than 10% of the cells, mainly by the human ES cells, and not by the feeder layer of mouse embryonic fibroblasts (MEF) (over 80% of the fluorescent cells had ES cell morphology and resided within the colony boundaries) (Figure 1, inset). To allow quantification and comparison of transfection efficiencies between protocols, a TK-firefly Renilla luciferase reporter gene (Dual Luc Reporter Assay Kit, Promega) was introduced into growing colonies of human ES cells, either by LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas) (performed according to the manufacturer's protocols). Cell samples were lysed (using the passive lysis buffer of the assay kit) and evaluated for the efficiency of transient transfection by measuring the relative activity of luciferase in respect to protein concentration (as determined by the Bradford method [BIO-RAD Protein Assay]) 48 hours after transfection. A clear difference between ExGen 500, FuGENE, LipofectAMINE Plus, and electroporation was apparent. Transfection with ExGen 500 seems to deliver DNA into human ES cells in an order of magnitude more efficiently than other reagents that we have examined (Figure 1).

Using the transfection protocol of ExGen 500, a *Rex1*-EGFP expression vector, which includes the neo selectable marker, was delivered into human ES cells. The following day, cells were trypsinized and replated on a feeder of inactivated MEF that was resistant to neomycin (MEF^{Neo+}), allowing the clonal propagation of transfected cells by G418 selection. At 14 days in culture, neomycin-resistant fluorescent colonies were isolated and propagated for several passages while maintaining their level of fluorescence (up to 13 passages), allowing the establishment of individual cell lines. In our experience, stable clones were derived in an efficiency of $\sim 10^{-5}$ of the transfected cells.

Of the various neo resistant colonies, we have established 10 cell lines, 4 of which were examined under different culture conditions (Figure 2). When grown on feeder cells in the presence of leukemia inhibitory factor (LIF) (to support undifferentiated growth), high expression of GFP was detected in the small and densely packed cells of the undifferentiated colony. The fluorescent emission overlaps well with the discrete margins of the colony and is absent in the periphery, where spontaneous differentiation takes place (Figure 2a). When the transfected human ES cells were induced to differentiate by growing as cell aggregates in suspension culture, fluorescence gradually declines, initially, in the outer surface of 4 day old simple

Figure 2

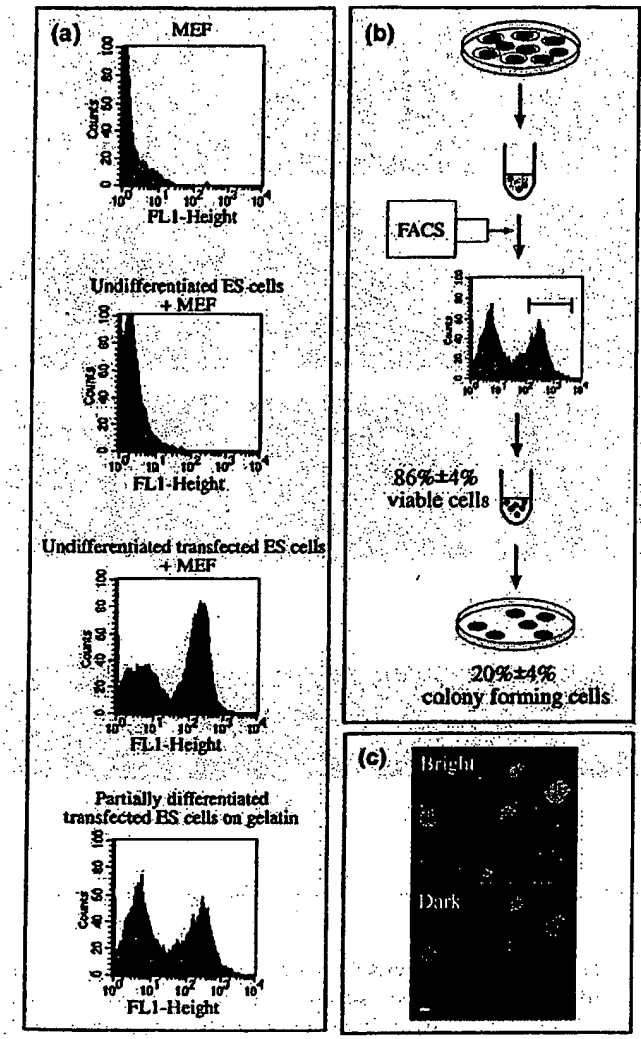


Isolation of human ES clones transfected with a marker for undifferentiated cells. **(a)** Human ES cells underwent stable transfection with EGFP fused to the murine *Rex1* minimal promoter sequence. The transfected ES cells and their differentiated cell derivatives are shown: simple embryoid body (sEB), and mature embryoid bodies (maEBs). The left and middle columns are photos of bright and dark fields, respectively. The right column is the overlay of the two photos. Note that only the undifferentiated cells are fluorescent. The fluorescent ES colony is surrounded by differentiated nonfluorescent cells. The simple EB is labeled only in the middle and not in the peripheral primitive endodermal cells [11]. Mature EBs are generally not fluorescent, and only very distinct areas in them are still fluorescent (probably residual undifferentiated cells). The scale bar indicates 100 μm. **(b)** The stable transfection of human ES cells with a constitutively expressed EGFP construct, driven by the mouse *PGK* promoter. Overlay photos of the dark on bright field of the transfected ES cells (ES) and their differentiated cell derivatives are shown: simple embryoid bodies (sEB), mature embryoid bodies (maEB), and differentiating embryonic cells derived from dissociated embryoid bodies (DE). Note that GFP is expressed by all cells, differentiated and undifferentiated, in the proliferating ES colony as well as by all cells of simple and mature EBs (including those in the outer layer of the sEB, where differentiation of primitive endoderm is taking place in the mouse EBs [11]). The scale bar indicates 100 μm.

EBs, where a layer of primitive endoderm was demonstrated in the mouse [11]. Later, if maintained to form mature EBs (20 days in suspension), the fluorescence practically ceases, apart from a few cores of undifferentiated cells (Figure 2a). This is in contrast to transfections of constructs driven by constitutively expressed promoters (*PGK*, phosphoglycerate kinase 1; and *CMV*, cytomegalovirus), in which expression of GFP was observed in both undifferentiated and differentiated cells of the colony (Figure 2b).

In an attempt to distinguish between populations of undifferentiated and differentiated human ES cells, we have analyzed the *Rex1*-EGFP transfected cell lines by FACS (Figure 3). Cell samples of MEF, undifferentiated human ES cells, and a mixture of undifferentiated and differentiated transfected cell lines were characterized according to their fluorescent emission. As expected, a clear difference in fluorescent intensity exists between the undifferentiated cultures of untransfected and transfected cell lines. In addition, when comparing EGFP-transfected human ES cells to their differentiated derivatives, a reduction in emission intensity is observed (Figure 3a). This shift in fluorescence emission represents a transition, from undifferentiated to differentiated, in the state of the cells. To allow the collection and selective propagation of the most fluorescent cells in the culture, three different GFP-expressing human ES cell lines (3–4 cell sample replicates per clone) were sorted by FACS (Figure 3b). Cell sorting was performed according to the background level of fluorescence that had been obtained by the analysis of untransfected human ES cells. The different cell samples were individually sorted for collection into tubes containing 25,000–50,000 cells each. By comparing the total cell count and the number of viable cells prior and following cell sorting (determined by trypan blue staining), we could show that the FACS procedure had no detrimental effect on cell viability, as 86% of the sorted cells were viable. Moreover, by plating the isolated cells on MEF^{Neo+} 10 cm^2 culture dishes and allowing their propagation in vitro, we demonstrated their ability to develop into undifferentiated fluorescent-labeled human ES colonies, with an efficiency of 20% ± 4% (n = 11) (ranging from 2% to 41%) (Figure 3b). In our procedure, many sorted cells were grown in the same culture dish, potentially allowing

Figure 3



FACS analysis and cell sorting of the transfected human ES cells. (a) Human ES cells transfected with *Rex1*-EGFP construct were analyzed by FACS according to the intensity of green fluorescence emission (FL1 height). Cell samples of MEF and undifferentiated human ES cells were used as controls. Fluorescent intensity between undifferentiated human ES cells, transfected human ES cells, and their differentiated cell culture derivatives (obtained by growth on gelatin-coated plates in the absence of LIF and bFGF) was then compared. The high-fluorescent intensity peak represents GFP positive cells, while the low-intensity peak represents background levels that may result either from autofluorescence or residual promoter activity. (b) Cell sorting of three GFP-expressing cell lines was performed by FACS. Following trypsin digestion, cell samples (3–4 replicates per clone) were evaluated for percentage of cell viability (84%) and sorted according to the intensity of green fluorescent emission. The collected cell samples (25,000–50,000) were redetermined for cell viability (86%) and replated on MEF^{Neo+} culture dishes (2,500–8,000 cells per dish). Following growth in vitro, cell culture dishes were inspected and recorded for total number of proliferating human ES colonies (20% ± 4% [n = 11]). (c) Photos of fluorescent-labeled proliferating human ES colonies (top, bright field; and bottom, dark field) obtained 4 days after cell sorting by FACS.

mutual support of growth and relatively high plating efficiency. This differs from the single cell dilution procedure by which the clonality of human ES cells was conferred [6]. After FACS sorting, the cells have a morphology indistinguishable from that seen before, but we have not yet tested them for pluripotency.

In our research, we have developed a stem cell selection approach in an attempt to facilitate maintenance of human ES cells in vitro. Currently, the available methods applied for this purpose involve the identification and isolation of single colonies under a dissecting microscope; however, these procedures are time consuming and labor intensive. As an alternative, we suggest a method for purifying undifferentiated cells by cell sorting the fluorescent-labeled cells from a mixed population. Similar selection of undifferentiated clones may be achieved by introducing into the cells a gene that enables drug selection, such as neo resistance gene, under the regulation of an ES-specific promoter [12]. By generating pure populations of undifferentiated cells, as described above, we should be able to avoid the loss of human ES cultures due to their spontaneous differentiation in vitro. Our system of introducing a cell-specific selectable marker into the genome of undifferentiated human ES cells provides a model for isolating specific cell types for transplantation from heterogeneous cell cultures obtained by induced differentiation. Similarly, such methods may be considered for eliminating human ES cells by negative selection prior to transplantation of differentiated cells, avoiding the risk of tumor induction.

The expression of *Rex1*-regulated reporter gene by the cells in the growing colony illustrates that these cells maintained their undifferentiated phenotype. In addition, the transfected cells can develop into undifferentiated colonies that maintain their ability to form EBs in vitro. These results support previous work that demonstrated the clonality of human ES cells [6] and the capacity of these homogenous cultures to differentiate into the three germ layers.

Finally, we report the first isolation of genetically engineered human ES cell lines and describe an efficient protocol for transfecting these cells. By introducing genetic modifications into their genome, we should be able to manipulate them in vitro and use them as vectors in cell-based therapies as well as for other biomedical and research purposes.

Materials and methods

Cell culture

Human ES cells (H9 [1], passage 40–50) were cultured on a Mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder layer (obtained from 13.5 day embryos) in 80% KnockOut DMEM medium (GIBCO-BRL), supplemented with 20% KnockOut SR (a serum-free formulation) (GIBCO-BRL), 1 mM glutamine (GIBCO-BRL), 0.1 mM β–mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (50 units/ml), Streptomycin (50 μg/ml), and 4 ng/ml basic fibroblast

growth factor (bFGF). The cells were grown in the presence of LIF (10^3 units/ml, GIBCO-BRL), although its necessity for supporting undifferentiated growth in human ES cells is currently unclear [1, 2]. The undifferentiated cell cultures were induced to differentiate in vitro into EBs by omitting LIF and bFGF from the growth media and allowing aggregation in petri dishes [3]. Following the formation of simple EBs by a 5 day cell aggregation step, cell masses were either trypsin dissociated and left to grow as a monolayer on fibronectin-creating cultures of differentiated embryonic (DE) cells [5] or further expanded in suspension and allowed to develop into 20 day old mature EBs (maEBs) (yielding cavitated and cystic EBs). In addition, we allowed some undifferentiated cells to undergo spontaneous differentiation as a monolayer by growing them on 0.1% gelatin-coated plates (Merck) in the absence of LIF and bFGF.

Plasmid construction

Rex1-EGFP and *PGK*-EGFP expression vectors were constructed by the deletion of the *CMV* promoter sequence from pEGFP-N1 (Clontech) and the insertion of either the mouse *Rex1* promoter sequence (700 bp) into the HindIII restriction site or the mouse *PGK* (phosphoglycerate kinase 1) promoter (515 bp) into the EcoRI and BamHI restriction sites. These constructs contained an SV40-driven neo selectable marker. The use of SV40 promoter in our system was sufficient to confer G418 resistance by driving the neo gene, although it was somewhat inefficient in mouse ES cells.

Transfection and establishment of transgenic cell lines

Fully expanded and undifferentiated human ES cells underwent stable transfection with *Rex1*-EGFP, *CMV*-EGFP, or *PGK*-EGFP plasmid DNA by the ExGen 500 transfection system (Fermentas). Transfection of human ES cells was carried out in 6-well trays on MEF, two days after plating, and was performed as described by the manufacturer's protocol. Specifically, 2 μg of plasmid DNA plus 10 μl of the transfecting agent ExGen 500 were added to ~3×10^5 cells in a final volume of 1 ml media per well. The cells were centrifuged at 280 × g for 5 min and incubated at 37°C in a moist chamber for an additional 45 min. Residuals of the transfecting agent were removed by washing the cells twice with PBS. The following day, the cells were trypsinized and ~5×10^5 were replated on each 10 cm culture dish containing inactivated MEF^{Neo+}. Two days following replating, G418 (200 ng/ml) was administered to the growth medium, allowing the selective propagation of transfected cells in culture. By day 14, neo resistant fluorescent-labeled colonies were identified by a fluorescent microscope (up to 10 colonies per plate). Using our constructs, over 80% of the neo resistant colonies were also GFP positive. Single transgenic colonies were picked by a micropipette, dissociated into small clumps of cells, and transferred into a 24-well culture dish on a fresh feeder of MEF^{Neo+}. The cells continuously proliferated in the presence of G418 and formed a large number of expanding undifferentiated colonies.

FACS analysis and cell sorting

FACS analysis of *Rex1*-EGFP-expressing cells was performed on a FACSCalibur system (Becton-Dickenson), according to their green fluorescent emission. Undifferentiated human ES cells were used to set the background level of fluorescence. Transfected cells, either undifferentiated (grown on MEF cells in the presence of LIF) or partially differentiated (obtained by growth on gelatin in the absence of LIF and bFGF) were analyzed for fluorescence intensity and compared to control cells.

GFP-expressing cell lines were sorted by FACS according to their fluorescence emission. Following trypsin digestion and centrifugation, cell pellets (~$2–5\times10^6$ cells from each cell line) were resuspended in PBS, filtered by a 70 μM cell strainer (Falcon), and divided into four different tubes, which were kept on ice under sterile conditions. Total cell counts and percent of viable cells were determined for each sample by 0.5% trypan blue staining (1:1 volume) prior to analysis and sorting by FACS. Cell samples were sorted for the collection of 25,000–50,000 cells in 50 ml conical tubes (Falcon) precoated with BSA (4% in PBS). Following centrifugation (5 min, 1000 rpm), the cells were resuspended in 0.5 ml PBS, analyzed for cell viability as described above, and plated on MEF^{Neo+} 10 cm culture dishes. Following 4 days in culture in the presence of G418 (200 ng/ml), the cell cultures were inspected under the microscope, and the total number of colonies per plate was recorded.

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Homologous recombination in human embryonic stem cells

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Homologous recombination applied to mouse embryonic stem (ES) cells has revolutionized the study of gene function in mammals[1–4]. Although most often used to generate knockout mice, homologous recombination has also been applied in mouse ES cells allowed to differentiate *in vitro*. Homologous recombination is an essential technique if human ES cells[5] are to fulfill their promise as a basic research tool. It also has important implications for ES cell–based transplantation and gene therapies. Significant differences between mouse and human ES cells have hampered the development of homologous recombination in human ES cells. High, stable transfection efficiencies in human ES cells have been difficult to achieve, and, in particular, electroporation protocols established for mouse ES cells work poorly in human ES cells[6]. Also, in contrast to their murine counterparts, human ES cells cannot be cloned efficiently from single cells, making it difficult to screen for rare recombination events[7]. Here we report an electroporation approach, based on the physical characteristics of human ES cells, that we used to successfully target *HPRT1*, the gene encoding hypoxanthine phosphoribosyltransferase-1 (HPRT1), and *POU5F1*, the gene encoding octamer-binding transcription factor 4 (Oct4; also known as POU domain, class 5, transcription factor 1 (POU5F1)).

The *HPRT1* gene is located on the X chromosome, so a single homologous recombination event leads to complete loss of function in XY cells. HPRT1-deficient cells can be selected based on their resistance to 2-amino-6-captopurine (6-TG), and thus the frequency of homologous recombination events is easy to estimate[8]. Because of these properties, HRPT1 played an important role in the initial development of homologous recombination in mouse ES cells[4,9]. We designed an *HPRT1*-targeting vector that contains a short homologous arm (1.9 kb) on the 5′ side of exon 7 and a long homologous arm (10 kb) on the 3′ side of exon 9, which deletes regions of the last three exons (Fig. 1A). A neomycin resistance (*neo*) cassette was inserted between the two homologous arms, and at the end of the 3′ homologous arm, the thymidine kinase gene (*tk*) was added to allow negative selection with gancyclovir.

For human ES cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates of about 10^{-5}; mouse ES cell electroporation procedures yield substantially lower rates[6]. Given the very low transfection rates previously reported for electroporation, we first tested two chemical transfection reagents (ExGen 500 versus FuGene-6) for homologous recombination of the *HPRT1* locus in human ES cells. Although clones were obtained using both transfection reagents—for ExGen and FuGene respectively, 130 versus 261 G418-resistant clones and 35 versus 61 gancyclovir-resistant clones—none of these were resistant to both G418 and 6-TG (*HPRT1*$^-$), indicating that none were the result of homologous recombination. These results are consistent with the observation that transfection using lipid (FuGene-6; Roche, Indianapolis, IN) and cationic (ExGen 500; Fermentas, Hanover, MD) reagents results in inefficient homologous recombination in other mammalian cell types, and that physical methods of introducing DNA are, in general, more effective[10].

Our failure to achieve homologous recombination with chemical transfection reagents led us to re-evaluate electroporation procedures for human ES cells. In our hands, electroporation using a typical mouse ES cell protocol[11] (220 V, 960 μF, electroporation in PBS) yielded a stable transfection rate of ~10^{-7}. Given current culture techniques, this frequency is too low to be practical for identifying rare homologous recombination events. As human ES cells are significantly larger than mouse ES cells (~14 μm versus ~8 μm), we tried electroporation parameters described for larger cells. Also, because our current culture conditions allow only about 1% of individual human ES cells to survive and form colonies when plated at low densities, we electroporated the ES cells in clumps, not as individual cells, and plated them out at high densities. Additionally, we electroporated the cells in an isotonic, protein-rich solution (standard cell culture medium), instead of PBS, at room temperature. Using this modified protocol, we were able to obtain stable, G418-resistant clones at transfection rates that were 100-fold (or more) higher than those attained with standard mouse ES cell electroporation procedures. After transfection of 1.5×10^7 cells with the linearized *HPRT1*-targeting vector, we obtained 350 G418-resistant clones. Of these, 50 were resistant to gancyclovir, and of these, 7 were also resistant to 6-TG, suggesting successful homologous recombination. Polymerase chain reaction (PCR) and Southern blotting (Fig. 1B) confirmed that homologous recombination had occurred in all of these 6-TG-resistant clones.

One of the uses of homologous recombination in human ES cells will be to generate 'knock-in' cell lines with a selectable marker introduced into a locus with a tissue-specific expression pattern. Such knock-ins will be useful, for example, to purify a specific ES cell–derived cell type from a mixed population[12,13]. To test this approach, we introduced two reporter genes into the Oct4-encoding gene *POU5F1* by homologous recombination. Oct4, which belongs to the POU (Pit, Oct, Unc) family of transcription factors[14], is expressed exclusively in the pluripotent cells of the embryo and is a central regulator of pluripotency[14,15]. We introduced two promoterless reporter-selection cassettes into the 3′ untranslated region (UTR) of *POU5F1*. The first cassette contained an internal ribosomal entry site (IRES) sequence of the encephalomyocarditis virus and the gene *EGFP*, encoding the enhanced green fluorescence protein (EGFP). The second cassette included the same IRES sequence and the gene *neo*, encoding neomycin resistance. The cassettes were flanked by two homologous arms (Fig. 2A). After electroporation of 1.5×10^7 human ES cells with the linearized targeting vector (Fig. 2A), we obtained 103 G418-resistant clones. PCR (Fig. 2B, left) and DNA Southern blotting (Fig. 2B, right) demonstrated that 28 of these clones (27%) were positive for homologous recombination. Using a second targeting vector with a longer 3′ homologous arm, we obtained a higher rate of homologous recombination, almost 40% (22 homologous clones out of 56 G418-resistant clones). Similar transfection experiments using FuGene-6 with the same *POU5F1*-targeting vector resulted in 11 G418-resistant clones, none of which resulted from homologous recombination.

Human ES cells with the *POU5F1* knock-in expressed EGFP (Fig. 2C), which turned off during differentiation. Both drug selection and flow cytometry to detect EGFP expression (Fig. 2D) allowed purification of undifferentiated ES cells from a mixed, partially differentiated cell population. These properties will make the knock-in cell lines useful for studying *POU5F1* gene expression during differentiation *in vitro* and for optimizing culture conditions for human ES cells.

Electroporation of human ES cells with a DNA construct containing a *neo* cassette under the control of the *tk* promoter yielded a stable

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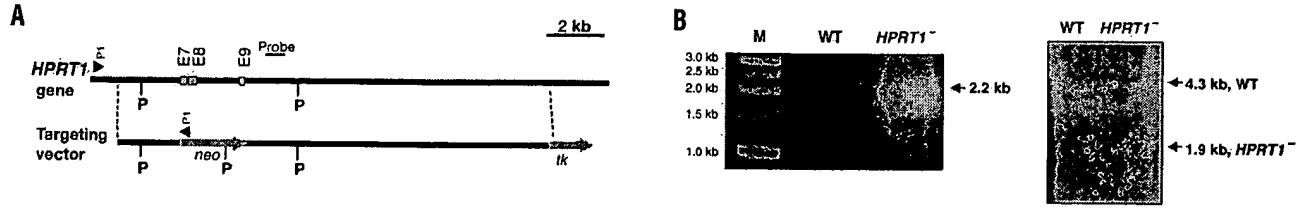

Figure 1. Targeted deletion of the last three exons of the *HPRT1* gene. (A) Partial structure of human *HPRT1* and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; P, *PstI*; P1, primer pair; *tk*, thymidine kinase gene. (B) PCR analysis of ES cell lines with P1 (left) and Southern blot analysis with dedicated probe and *PstI* digest (right). *HPRT1*⁻, knockout; WT, wild-type cells; M, marker.

transfection rate of 5.6×10^{-5}, giving an estimated 26:1 ratio of stable transfected clones to homologous recombination events for the first *POU5F1* construct. Similarly, for transfection of the *HPRT1* vector, the ratio of G418-resistant clones to HPRT1⁻ clones was 50:1. These targeting ratios for both HPRT1 and Oct4 are comparable to those observed for mouse ES cells[16], and suggest that although successful transfection strategies differ between human and mouse ES cells, the frequency of homologous recombination itself may be similar. However, it will be important to determine whether this similarity of rates between human and mouse ES cells holds true for genes not expressed in ES cells.

Homologous recombination in human ES cells will be important both for elucidating gene function *in vitro* and for modifying specific ES cell–derived tissues for therapeutic applications in transplantation medicine. For therapeutic applications, controlled modification of specific genes should be useful for purifying specific ES cell–derived differentiated cell types from a mixed population, for altering the antigenicity of cells, and for giving cells new properties (such as viral resistance) to combat specific diseases. Homologous recombination in human ES cells might also be used for recently described approaches combining therapeutic cloning with gene therapy[17]. Modifying specific genes for *in vitro* studies will be important for learning more about the pathogenesis of diseases for which mouse models have proven inadequate. For example, Hprt1-deficient mice do not show a phenotype similar to Lesch-Nyhan syndrome, the condition that results from HPRT1 deficiency in humans[18]. *In vitro* neural differentiation of HPRT1⁻ human ES cells or transplantation of ES cell–derived neural tissue to an animal model[19] could help clarify the pathogenesis of Lesch-Nyhan syndrome. Indeed, homologous recombination and human ES cells offer a promising approach for understanding the function of any human gene, and this approach will be particularly important for human genes that differ in clinically significant ways from the corresponding mouse genes.

Experimental protocol

***HPRT1* knockout.** The gene-targeting vector was constructed by replacement of the last three exons (exon 7, 8, and 9) of the *HPRT1* gene with a *neo* cassette under the control of the *tk* promoter. This cassette is flanked in the 5′ direction by a 10 kb homologous arm and in the 3′ direction by a 1.9 kb homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. One week before electroporation, cells were plated onto Matrigel (Becton Dickinson, San Jose, CA) and cultured with fibroblast-conditioned medium[20]. To remove colonies as intact clumps, human ES cell cultures were treated with collagenase

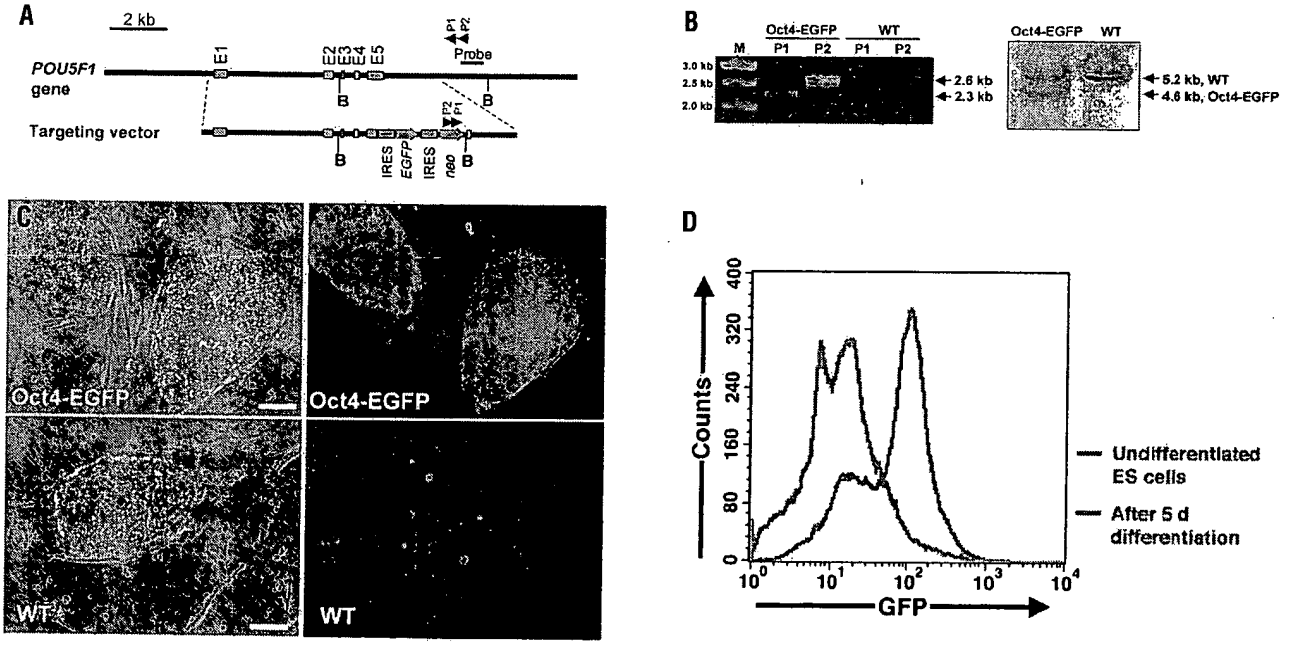

Figure 2. Targeting of an IRES-*EGFP*-IRES-*neo* cassette into the 3′ UTR of the gene *POU5F1*, which encodes Oct4. (A) Partial structure of the human *POU5F1* gene and the gene-targeting vector. 3′ probe for Southern blot analysis is shown. E, exon; B, *Bam*HI; P1 and P2, primer pairs 1 and 2. (B) PCR analysis of ES cell lines with P1 and P2 (left) and Southern blot analysis with dedicated probe and *Bam*HI digest (right). *POU5F1-EGFP*, heterozygous knock-in; WT, wild-type cells; M, marker. (C) Fluorescence microscopy (right) and phase-contrast microscopy (left) of *POU5F1* knock-in and wild-type colonies. Bar, 25 μm. (D) Flow cytometry of *POU5F1* knock-in undifferentiated (EGFP-positive) ES cells (blue) and their differentiated derivates after 5 d of differentiation (red).

IV (1 mg/ml; Invitrogen, Carlsbad, CA) for 7 min, washed with medium, and resuspended in 0.5 ml culture medium ($1.5–3.0 \times 10^7$ cells). Just before electroporation, 0.3 ml PBS (Invitrogen) containing 40 μg linearized targeting vector DNA was added. Cells were then exposed to a single 320 V, 200 μF pulse at room temperature using the BioRad Gene Pulser II (0.4 cm gap cuvette; BioRad, Hercules, CA). Cells were incubated for 10 min at room temperature and were plated at high density on one 10 cm culture dish coated with Matrigel. G418 selection (50 μg/ml, Invitrogen) was started 48 h after electroporation. After one week, G418 concentration was doubled and 6-TG selection (1 mM; Sigma, St. Louis, MO) was started. After three weeks, surviving colonies were analyzed individually by PCR using primers specific for the *neo* cassette and for the *HRPT1* gene just upstream of the 5′ homologous region, respectively. PCR-positive clones were rescreened by Southern blot analysis using *Pst*I-digested DNA and a probe on the 3′ side of the *neo* cassette.

***POU5F1* knock-in.** The gene-targeting vector was constructed by insertion of an IRES-*EGFP*, an IRES-*neo*, and an SV40 polyadenylation sequence (approximately 3.2 kb) into the 3′ untranslated region of the fifth exon of the human *POU5F1* gene. This cassette is flanked in the 5′ direction by a 6.3 kb homologous arm and in the 3′ direction by a 1.6 kb (6.5 kb in an alternative targeting vector) homologous arm. Isogenic homologous DNA was obtained by long-distance genomic PCR and subcloned. H1.1 human ES cells were cultured as described[5]. When an alternative targeting vector with a longer (6.5 kb) 3′ homologous arm was used, the rate of homologous recombination increased to almost 40% (22 homologous clones out of 56 stable clones).

Flow cytometry. Before flow cytometry, ES cell differentiation was induced by incubating the cells for 5 d in unconditioned medium on Matrigel. ES cells were treated with trypsin-EDTA and washed with PBS (both from Invitrogen). Dead cells were excluded from analysis by forward- and side-scatter gating. Samples were analyzed using a FACScan (Becton Dickinson) flow cytometer and Cellquest software (Becton Dickinson). A minimum of 50,000 events was acquired for each sample.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Site-specific cassette exchange and germline transmission with mouse ES cells expressing ϕC31 integrase

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Currently two site-specific recombinases are available for engineering the mouse genome: Cre from P1 phage[1,2] and Flp from yeast[3,4]. Both enzymes catalyze recombination between two 34–base pair recognition sites, *lox* and *FRT*, respectively, resulting in excision, inversion, or translocation of DNA sequences depending upon the location and the orientation of the recognition sites[5,6]. Furthermore, strategies have been designed to achieve site-specific insertion or cassette exchange[7–10]. The problem with both recombinase systems is that when they insert a circular DNA into the genome (*trans* event), two *cis*-positioned recognition sites are created, which are immediate substrates for excision. To stabilize the *trans* event, functional mutant recognition sites had to be identified[8–12]. None of the systems, however, allowed efficient selection-free identification of insertion or cassette exchange. Recently, an integrase from *Streptomyces* phage ϕC31 has been shown to function in *Schizosaccharomyces pombe*[13] and mammalian[14,15] cells. This enzyme recombines between two heterotypic sites: *attB* and *attP*. The product sites of the recombination event (*attL* and *attR*) are not substrates for the integrase[16]. Therefore, the ϕC31 integrase is ideal to facilitate site-specific insertions into the mammalian genome.

Here we demonstrate that the ϕC31 integrase system is compatible with embryonic stem (ES) cell–mediated genomic alterations in the mouse and is particularly useful to achieve site-specific transgene insertions or

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Exhibit D

Hematopoietic colony-forming cells derived from human embryonic stem cells

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Human embryonic stem (ES) cells are undifferentiated, pluripotent cells that can be maintained indefinitely in culture. Here we demonstrate that human ES cells differentiate to hematopoietic precursor cells when cocultured with the murine bone marrow cell line S17 or the yolk sac endothelial cell line C166. This hematopoietic differentiation requires fetal bovine serum, but no other exogenous cytokines. ES cell-derived hematopoietic precursor cells express the cell surface antigen CD34 and the hematopoietic transcription factors TAL-1, LMO-2, and GATA-2. When cultured on semisolid media with hematopoietic growth factors, these hematopoietic precursor cells form characteristic myeloid, erythroid, and megakaryocyte colonies. Selection for CD34$^+$ cells derived from human ES cells enriches for hematopoietic colony-forming cells, similar to CD34 selection of primary hematopoietic tissue (bone marrow, umbilical cord blood). More terminally differentiated hematopoietic cells derived from human ES cells under these conditions also express normal surface antigens: glycophorin A on erythroid cells, CD15 on myeloid cells, and CD41 on megakaryocytes. The *in vitro* differentiation of human ES cells provides an opportunity to better understand human hematopoiesis and could lead to a novel source of cells for transfusion and transplantation therapies.

Embryonic stem (ES) cells are pluripotent cells derived from preimplantation embryos. ES cells have the ability to be maintained in culture indefinitely as undifferentiated cells, yet they are capable of forming more differentiated cell types. Because of these properties, mouse ES cells have been instrumental in gaining a better understanding of mammalian development. In studies of hematopoiesis, investigators have used mouse ES cells to derive various hematopoietic lineages *in vitro* either by formation of "embryoid bodies" (1, 2), coculture with stromal cell lines (3, 4), or culture on collagen-coated plates (5). These studies have used gene expression, cell phenotype, and functional studies to define sequential stages of hematopoietic cell development.

In contrast to work on mouse hematopoietic development, studies of human hematopoiesis have been confined to the use of primary hematopoietic tissue such as bone marrow, peripheral blood, or umbilical cord blood as the starting cell population. The reliance on these heterogeneous tissue samples that are difficult to sustainably expand *in vitro* has hindered progress in understanding human hematopoiesis. Work on human hematopoiesis typically uses cell surface antigens (such as CD34) to identify putative hematopoietic stem cell (HSC) population(s) within the mixed cell population, and cell sorting methods are used to enrich for the cells of interest (6, 7). Recently, fluorescent dyes that can bind DNA (such as Hoechst 33342) have proven useful in the differential isolation of putative HSCs (8). Although these methods have provided a great deal of information about HSC biology and have facilitated clinical hematopoietic cell transplantation, several important questions remain. For example, recent studies have shown that some CD34$^-$ cells also can display HSC properties, including long-term growth, differentiation, and self-renewal when injected into immunodeficient mice (8–11). Moreover, some cells derived from nonhematopoietic tissue appear to have HSC potential (12, 13). The interrelationship between these varying sources and phenotypes of HSCs remains unclear.

Human ES cells (14, 15) provide a unique, homogeneous, unlimited starting population of cells for studying human hematopoiesis. Human ES cells can be cultured for at least 300 population doubling times without observed senescence, while continuing to maintain normal karyotypes, telomere lengths, and pluripotency. Moreover, these cells can be cloned from a single cell without loss of pluripotency (16). Human ES cells give rise to differentiated cells and tissues from all three embryonic germ layers when allowed to form teratomas in immunodeficient mice or when induced to form embryoid bodies *in vitro* (14, 17). Mouse and human ES cells differ in morphology, population doubling time, and growth factor requirements. Undifferentiated mouse ES cells, for example, can be maintained as undifferentiated "feeder-independent" cells if growth factors such as leukemia inhibitory factor (LIF) or related cytokines are added to the media (1). If human ES cells are grown without feeder cells, but in the presence of LIF, they either differentiate or die (14, 15). Given the unexpected differences in the control of the undifferentiated proliferation of mouse and human ES cells, similar significant differences could exist in the specific factors that direct their differentiation.

Here, we show that coculture of human ES cells with certain stromal cell lines derived from mouse hematopoietic tissue (yolk sac and bone marrow) leads to differentiation into hematopoietic cells. These cells express both cell surface antigens and transcription factors characteristic of cells in primary human hematopoietic tissue. Moreover, hematopoietic cells are present in these differentiating cocultures that can generate myeloid, erythroid, and megakaryocyte colonies *in vitro*, and the colonies obtained appear identical to those produced from human adult bone marrow cells.

Methods

Culture of ES Cells. The human ES cell lines H1, H1.1, and H9.2 were derived and maintained as described (14, 16), except that the undifferentiated ES cells were grown in serum-free conditions. Human ES cells were maintained as undifferentiated cells by coculture with irradiated (25 Gy) mouse embryonic fibroblast (MEF) cells in media consisting of DMEM/F12 (GIBCO/BRL) supplemented with 15% KnockOut SR serum replacer

Abbreviations: ES, embryonic stem; HSC, hematopoietic stem cell; MEF, mouse embryonic fibroblast cell; CFC, colony-forming cell; RT-PCR, reverse transcriptase–PCR; PE, phycoerythrin; CFU, colony-forming unit; CFU-Mk, CFU-megakaryocyte; CFU-M, CFU-macrophage; CFU-G, CFU-granulocyte; CFU-GM, CFU-macrophage/granulocyte.

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(GIBCO/BRL), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids (all from GIBCO/BRL), and 4 ng/ml basic fibroblast growth factor (R & D Systems). ES cells grown under these conditions will begin to show evidence of differentiation after ≈10 days; therefore, ES cells were passaged approximately weekly to maintain undifferentiated growth. To promote hematopoietic differentiation, the human ES cells were cocultured with either the mouse bone marrow stromal cell line S17 (18) (gift of Kenneth Dorshkind, University of California, Los Angeles) or the mouse yolk-sac endothelial cell line C166 (19). S17 and C166 cells were irradiated (30 Gy). Media to support differentiation consisted of DMEM (GIBCO/BRL) supplemented with 20% FBS (from either HyClone or Gencyte, Buffalo, NY), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids. During differentiation, media were changed every 2–3 days.

Flow Cytometry Analysis. Undifferentiated H1 cells were washed with Ca^{2+} and Mg^{2+}-free PBS and dissociated with 0.05% trypsin/0.53 mM EDTA (GIBCO/BRL) for 5–10 min before washing and staining with FACS media consisting of PBS supplemented with 2% FBS and 0.1% sodium azide. To analyze cell surface antigen expression on H1 cells allowed to differentiate as above (H1/S17 and H1/C166 cells), the differentiated cell mixture was dissociated with 1 mg/ml collagenase IV (GIBCO/BRL) and 0.05% trypsin/0.53 mM EDTA supplemented with 2% chick serum (GIBCO/BRL). Dissociated cells were filtered through 85-μm nitex mesh to remove remaining clumps. The single cell suspension was aliquoted and stained with either isotype control or antigen-specific antibodies. Unconjugated isotype control antibodies IgG3 and IgM (Sigma) and directly conjugated isotype control antibodies IgG1-FITC and IgG1-phycoerythrin (PE) (PharMingen) were used. Unconjugated antigen-specific antibodies against SSEA-1 (IgM) and SSEA-4 (IgG3) (Developmental Studies Hybridoma Bank, Iowa City) were detected with a FITC-labeled goat anti-mouse IgG and IgM antibody (Caltag, Burlingame, CA). Other conjugated antibodies were used: CD34-FITC, CD45-PE, CD31-PE, CD38-PE, CD90-FITC, CD117-PE, CD15-FITC, class I-FITC (all IgG1, all from PharMingen), CD133/1-PE (IgG1) (Miltenyi Biotec, Auburn, CA), and glycophorin A-PE (IgG1) (Immunotech, Miami, FL). Cells were analyzed live (without fixation) by using propidium iodide to exclude dead cells on a FACScan (Becton Dickinson) with either PC-LYSIS or CELLQUEST software.

Magnetic Column (MACS) Separation. Selection of $CD34^+$ cells was done by labeling the H1/S17 cells with the anti-CD34 antibody QBEND/10 followed by a magnetically labeled secondary antibody (Miltenyi Biotec). The magnetically labeled cells were separated into $CD34^+$ and $CD34^-$ populations with a mini-MACS column (Miltenyi Biotec). CD34 enrichment was confirmed by flow cytometry analysis using a different anti-CD34 antibody (PharMingen).

Hematopoietic Colony Assays. H1/S17 cells, H1/C166 cells, or H1/MEF cells were cultured for the indicated number of days before harvesting and making a single cell suspension as above. Hematopoietic colonies were demonstrated by growing these cells in Methocult GF+ media (StemCell Technologies, Vancouver) consisting of 1% methylcellulose, 30% FBS, 1% BSA, 50 ng/ml stem cell factor, 20 ng/ml granulocyte–macrophage colony-stimulating factor, 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml granulocyte colony-stimulating factor, and 3 units/ml erythropoietin. Cells were aliquoted in duplicate samples at $1–2 \times 10^5$ cell per plate. After 14 days the plates were scored for colony-forming units (CFUs) according to standard criteria (20, 21). To demonstrate CFU-megakaryocyte (CFU-Mk) colonies, the H1/S17 cells were cultured on chamber slides in MegaCult-C media (StemCell Technologies) consisting of 1.1% collagen, 1% BSA, 10 μg/ml bovine pancreatic insulin, 200 μg/ml human transferrin, 2 mM L-glutamine, 0.1 M β-mercaptoethanol, 50 ng/ml thrombopoietin, 10 ng/ml IL-6, and 10 ng/ml IL-3. The Megacult-C media were supplemented with 40 μg/ml low density lipoproteins (Sigma) as recommended by the manufacturer. After 10–14 days, the cells were fixed, dried, and stained with an anti-CD41 (GPIIb)-specific antibody or isotype control antibody, followed by an alkaline phosphatase-conjugated secondary antibody and visualization with Fast Red/Naphthol staining according to the manufacturer's instructions. CFU-Mk cells were identified by red staining.

Cellular morphology and enzyme expression were examined by plucking individual colonies with a pulled Pasteur pipette and spinning onto glass slides by using a Cytospin 2 (Shandon, Pittsburgh). Cells either were stained with Diff-Quik (a modified Wright-Giemsa stain, Dade Behring, Miami) or stained for esterase-containing cells with Naphthol AS-D Chloroacetate esterase and α-Naphthyl acetate esterase (Sigma).

Reverse Transcriptase–PCR (RT-PCR) Analysis. Cells used for initial RT-PCR studies were: H1 cells allowed to differentiate on S17 cells (H1/S17), H1 cells allowed to differentiate on MEFs (H1/MEF day 17), H1 cells on MEFs for 6 days and harvested before differentiation was seen (H1/MEF day 6), irradiated S17 cells alone, irradiated MEF cells alone, and the erythroleukemia cell line K562 (American Type Culture Collection). Adherent cells were harvested with 1 mg/ml collagenase IV, washed with PBS, and pelleted. Total RNA was extracted by using a RNeasy mini kit (Qiagen, Valencia, CA) with homogenization with a Qiashredder (Qiagen) according to the manufacturer's instructions. Total RNA was quantified by UV spectrophotometer and 1 μg was used for each RT reaction. For time-course experiments, 0.5 μg RNA was used for each RT sample. RT reactions were done by using Omniscript RT (Qiagen) according to the manufacturer's instructions. Duplicate samples with and without addition of RT enzyme were done for all studies to control for contaminating genomic DNA. RT reactions were primed by using oligo(dT) primers (Promega), and 20 units RNase inhibitor was added to each reaction (Promega). PCRs were done with HotStarTaq (Qiagen) using 2 μl of RT product per reaction according to the manufacturer's instructions. PCR conditions consisted of: 15 min at 95°C (hot start), 25–40 cycles (actual number noted below) of: 94°C for 1 min, annealing temperature (T_a, noted below) for 1 min, 72°C for 1 min. A final 10-min extension at 72°C was done at the end. Oligonucleotide-specific conditions were: TAL-1, 40 cycles, T_a 53°C; GATA-2: 31 cycles, T_a 53°C; Flk-1, 35 cycles, T_a 53°C; LMO-2, 40 cycles, T_a 53°C; and β-actin, 25 cycles, T_a 58°C. Products were analyzed on 1.5% agarose gel and visualized with ethidium bromide staining. DNA sequencing was done to confirm bands corresponded with the appropriate human genes. Oligonucleotide primers were: TAL-1 (331 bp), forward, 5′-ATGGTGCAGCTGAGTCCTCC-3′, reverse, 5′-TCTCATTCTTGCTGAGCTTC-3′; GATA-2 (242 bp), forward, 5′- AGCCGGCACCTGTTGTGCAA-3′, reverse, 5′-TGACTTCTCCTGCATGCACT-3′; Flk-1 (537 bp), forward, 5′-ATGCACGGCATCTGGGAATC-3′, reverse, 5′-GCTACTGTCCTGCAAGTTGCTGTC-3′; LMO-2 (289 bp), forward, 5′-GGATCCTGCCGGAGAGACTATCTC-3′, reverse, 5′-GAATTCAGTGAACACCTCCGCAAA-3′; and β-actin, (838 bp), forward, 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′, reverse, 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′.

Results

Hematopoietic Differentiation of Human ES Cells. The majority of these experiments were done by using the human ES cell line H1 (14). These cells were maintained in the undifferentiated state

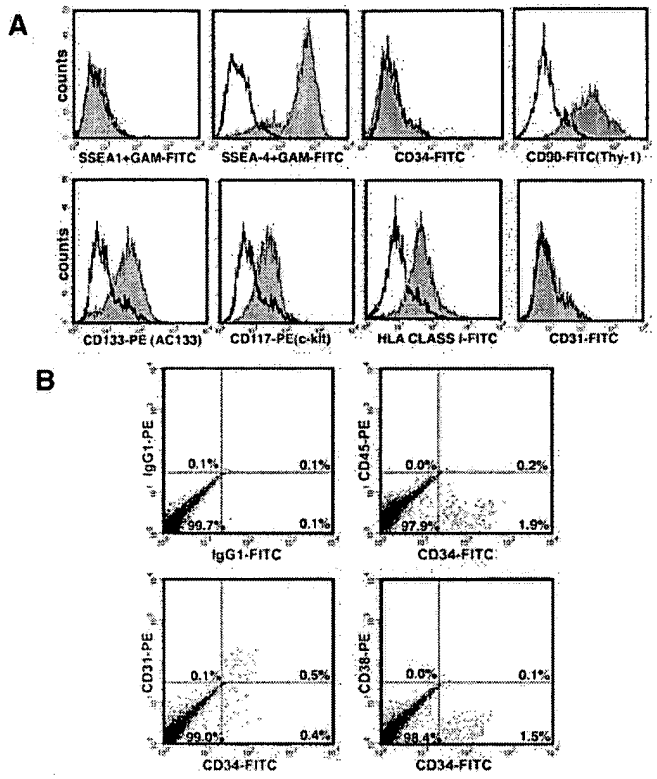

Fig. 1. Flow cytometric analysis of undifferentiated human ES (H1) cells and differentiated H1 cells. (*A*) Undifferentiated H1 cells analyzed by single-color flow cytometry. Appropriate isotype control antibody is demonstrated by line and indicated antibody by filled plot. SSEA-1 and SSEA-4 were unconjugated antibodies and a secondary FITC-conjugated goat anti-mouse (GAM) antibody was used. All other antibodies were directly conjugated to flurochrome. (*B*) H1 cells allowed to differentiate on S17 cells (H1/S17 cells) analyzed by two-color flow cytometry. Percentages of positive cells are indicated in each quadrant.

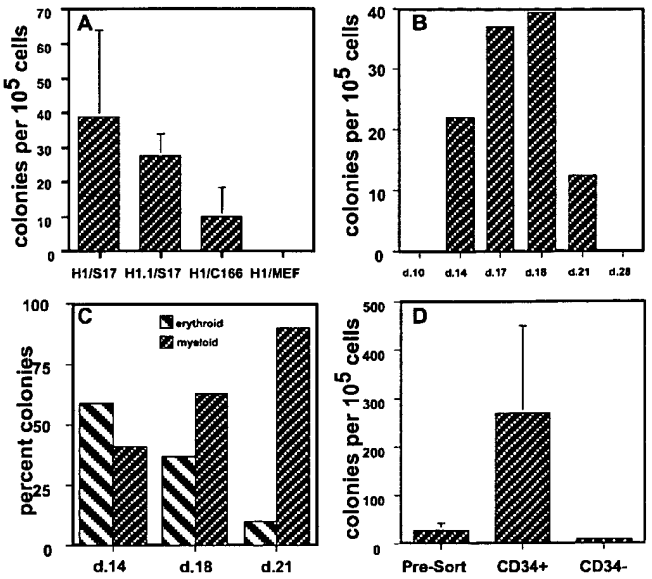

Fig. 2. Methylcellulose hematopoietic colony-forming assays. (*A*) Production of CFCs from human ES cells. H1/S17 cells, H1.1/S17, H1/C166 cells, and differentiated H1/MEF cells were harvested after 14–20 days of culture, placed in methylcellulose-based media supplemented with hematopoietic growth factors, and scored for total hematopoietic colonies after 14 days. Results are mean ± SE of seven trials with H1/S17, four trials with H1.1/S17, four trials with H1/C166, and three trials with H1/MEF. Data are presented as colonies per 10^5 cells harvested from the differentiated H1 cultures. (*B*) Time course of H1/S17 cell differentiation into hematopoietic CFCs. H1 cells cocultured with S17 cells for the indicated number of days before colony assay. (*C*) Percent of erythroid (burst-forming unit-erythroid) and myeloid (CFU-GM, CFU-M, and CFU-G) colonies derived from H1/S17 cells harvested at day indicated. (*D*) $CD34^+$ H1/S17 cells are enriched for CFCs. Unsorted H1/S17 cells and H1/S17 cells sorted for $CD34^+$ cells and $CD34^-$ cells by magnetic column were placed in hematopoietic colony assay. These results are mean ± SE of three separate trials.

by coculture on irradiated MEF "feeder cells" in serum-free media. Flow cytometric analysis of undifferentiated H1 cells demonstrates they are SSEA-1^-, SSEA-4^+, as previously demonstrated by immunohistochemical staining (14) (Fig. 1*A*). Here, we further characterized these human undifferentiated ES cells as expressing CD90 (thy-1), CD133 (AC133), and CD117 (c-*kit*). However, H1 cells fail to express CD34, CD31, CD45, and CD38 (Fig. 1*A* and data not shown). Interestingly, CD90, CD133, and CD117 (c-*kit*) are well recognized to be present on HSCs, and recently CD133 was identified on purified human neural stem cells (7, 22, 23).

To promote hematopoiesis, the undifferentiated H1 cells were cocultured with irradiated S17 cells (originally derived from mouse bone marrow; ref. 18), or with C166 cells (originally derived from embryonic day 12 mouse yolk sac; ref. 19). The media contained 20% FBS, but no other exogenously added cytokines or growth factors. Both the S17 and C166 cell lines have been shown to support the growth of bone marrow-derived hematopoietic progenitor cells (24, 25). After 3–5 days in culture under these conditions, the H1 cells differentiated into a variety of cell types. Within these areas of differentiation were regions of cobblestone-type cells and other areas of small, round loosely adherent cells. The appearance of these cells is reminiscent of early hematopoietic cells derived from other sources (7).

Initially, to characterize potential hematopoietic cells, the H1 cells allowed to differentiate on either S17 cells (H1/S17 cells) or C166 cells (H1/C166 cells) for 17 days were analyzed by flow cytometry (Fig. 1*B*). Approximately 1–2% of the differentiated H1 cells were shown to be $CD34^+CD38^-$, consistent with the phenotype of early hematopoietic cells (7, 26). Interestingly, roughly 50% of the $CD34^+$ cells also expressed CD31 on the cell surface. Other studies have shown that CD34 and CD31 can be coexpressed on both HSCs and endothelial cells, and both cell types are thought to be derived from the same hemangioblast precursor cells (27–29). Therefore, it is possible that endothelial cells or endothelial precursors are also present within these cultures of differentiated H1 cells. These $CD34^+CD38^-$ cells also were found to be $CD45^-$ (Fig. 1*B*). Although CD45 is commonly expressed on mature hematopoietic cells, expression of CD45 on HSCs and hematopoietic colony-forming cells (CFCs) is unclear. Studies of both human and murine hematopoiesis have identified $CD45^-$ hematopoietic precursors. This includes work on differentiated mouse ES cells and day 9.5 mouse embryonic yolk sac that demonstrate hematopoietic CFCs from $CD45^-$ cell populations (5).

Hematopoietic Colony Assays. Cells that form hematopoietic colonies (so-called CFUs or CFCs) represent a stage of hematopoietic differentiation between HSCs and more terminally differentiated cells (such as erythrocytes, granulocytes, monocytes, or platelets). These CFCs are identified by culturing them in a semisolid media (typically methylcellulose or agar) supplemented with cytokines that promote the localized expansion and differentiation of hematopoietic cells in discrete colonies. In methylcellulose assays, on average, H1/S17 cells gave rise to 30.4 colonies per 10^5 input cells, and H1/C166 gave rise to 4.3 colonies per 10^5 input cells (Fig. 2*A*). H1/S17 cells produced CFCs after 14 days of coculture (but not at earlier times),

produced a maximal number of CFCs at 17–18 days, and produced no CFCs at 28 days (Fig. 2*B*). This finding demonstrates the transient nature of hematopoietic differentiation within this system and suggests that long-term self-renewal of HSCs is not supported by this stromal coculture method. The clonally derived human ES cell lines H1.1 and H9.2 gave similar results when allowed to differentiate on S17 cells (Fig. 2*A* and data not shown). Importantly, none of the following conditions lead to generation of CFCs: undifferentiated H1 cells (harvested after 6 days on MEFs without evidence of differentiation), H1 cells allowed to differentiate on MEF cells for 17 days (H1/MEF), H1 cells allowed to differentiate on S17 cells in serum-free media, and S17 cells or C166 stromal cells alone (Fig. 2*A* and data not shown). H1/S17 cells and H1/C166 cells gave rise to both erythroid and myeloid (nonerythroid) colonies (Fig. 2*C*). Interestingly, after 14 days of differentiation H1/S17 cells produced mostly erythroid CFCs, whereas after 21 days of differentiation H1/S17 cells produced mostly myeloid CFCs (Fig. 2*C*).

Because CD34 is the best identified surface antigen expressed on hematopoietic precursor cells, the H1/S17 cells were enriched for $CD34^+$ cells by magnetic selection. Here, the CD34-enriched cells gave rise to on average 270 colonies per 10^5 H1/S17 cells and the CD34-depleted cells gave rise to only 10 colonies per 10^5 H1/S17 cells (Fig. 2*D*). Therefore CD34 selection markedly enriched CFCs compared with the unselected H1/S17 cells, whereas the CD34-depleted cell population was reduced in CFCs. Because the CD34-depleted cells still contained ≈0.5% $CD34^+$ cells (postdepletion), the CFCs from the CD34-depleted cells may have come from either $CD34^-$ cells or contaminating $CD34^+$ cells.

The colonies formed in methylcellulose had the same highly characteristic morphologies of colonies derived from human bone marrow cells placed in similar culture conditions (Fig. 3 *A–E*)(20, 21). The phenotypes of the CFCs included CFU-macrophage (CFU-M), CFU-granulocyte, mixed CFU-macrophage/granulocyte (CFU-GM), burst-forming unit-erythroid, and CFU-erythroid. Occasional experiments also demonstrated mixed CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), an early multipotent progenitor cell. CFU-Mks were demonstrated by culture of the H1/S17 cells in a collagen based-media designed to support CFU-Mk growth. The CFU-Mk were specifically identified by immunostaining with an antibody against CD41 (GPIIb of the GPIIb/IIIa complex), specific for megakaryocytes and platelets (Fig. 3*E*). Staining of the CFU-M- and CFU-GM-derived colonies with nonspecific esterase demonstrated granules typical of these lineages (Fig. 3*F*). Mature neutrophils also could be identified within the CFU-GM-derived cells by their typical nuclear morphology (Fig. 3*F*). Flow cytometric analysis of cells within these colonies demonstrates expression of surface antigens typical of normal human blood cells. These cells are $CD45^+$, HLA class I^+, and $CD34^-$. The erythroid cells express glycophorin A, and the myeloid cells express CD15 (Fig. 4).

Hematopoietic Gene Expression. To further characterize the H1 cells differentiated to hematopoietic cells by coculture with S17 cells (H1/S17 cells), we examined genes known to be expressed at an early stage of hematopoietic differentiation by using RT-PCR. H1/S17 cells expressed mRNA for TAL-1 and GATA-2, confirming the presence of early hematopoietic cells uniquely within this population (Fig. 5*A*). The vascular endothelial growth factor receptor Flk-1 and the transcription factor LMO-2 were expressed in undifferentiated H1 cells, H1/S17 cells, and H1/MEF cells. This finding suggests that Flk-1 and LMO-2 may have important roles in cells other than hematopoietic cells, including undifferentiated ES cells. The S17 and MEF feeder cells alone do not express any of these genes (Fig. 5 and data not shown). Time-course analysis found expression of TAL-1 and GATA-2 as early as day 7, before the appearance of CFCs. However, expression of GATA-2 was not detectable after day 21, corresponding to loss of CFC generation (Fig. 5*B*).

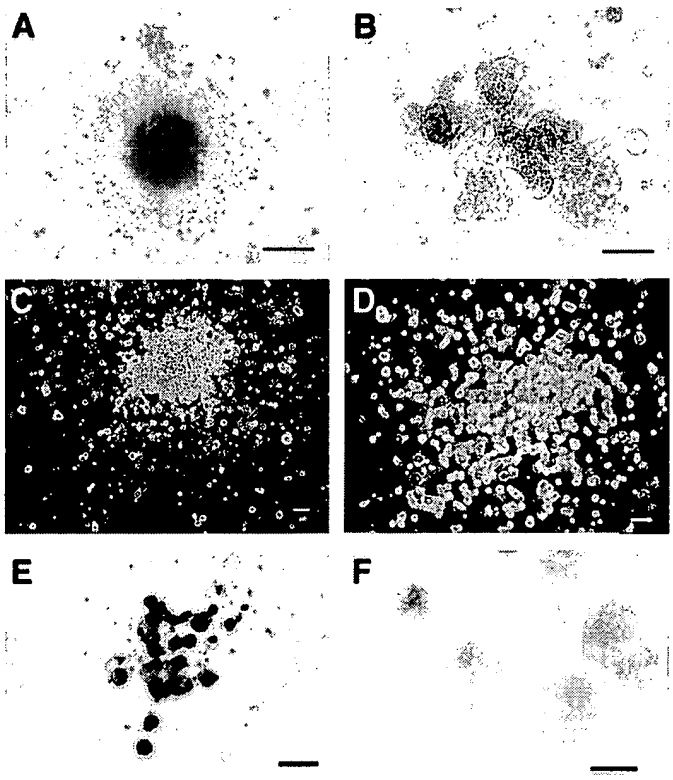

Fig. 3. Photographs of hematopoietic colonies and cells derived from H1/S17 cells. H1 cells allowed to differentiate on S17 cells for ≈17 days, harvested, and allowed to form colonies in semisolid media for 14 days before scoring colony phenotypes. (*A*) CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Colony of mixed erythroid and myeloid cells. (*B*) Burst-forming unit-erythroid. Large unstained, red (hemoglobin) colony. (*C*) CFU-GM, unstained myeloid colony. (*D*) CFU-M, unstained myeloid colony, less dense than CFU-GM colony. (*E*) CFU-Mk. Colony of cells stained with platelet/megakaryocyte-specific antibody against CD41 (GPIIb/IIIa) with alkaline phosphatase-conjugated secondary antibody and Fast Red/naphthol reagent to provide red stain. (*F*) Cytospin of CFU-GM cells demonstrating granulocytes with esterase-positive red granules. (Scale bars: *A–D*, 100 μm; *E*, 40 μm; *F*, 20 μM.)

Because the ES cell-derived erythroid colonies could potentially express globin genes from any stage of development (embryonic, fetal, or adult), we used RT-PCR to evaluate this gene expression. RNA was prepared from erythroid colonies harvested from methylcellulose culture of differentiated H1/S17 cells and compared with erythroid colonies formed by normal adult bone marrow. As expected, the adult bone marrow-derived colonies expressed ample α and β RNA, as well as some γ and δ RNA. In contrast, the H1/S17-derived colonies also expressed α, β, and δ globin, but did not express fetal γ globin. No embryonic (ε or ζ) globin gene expression was detected (data not shown). These results show that the ES cell-derived erythoid cells can express mature, adult-type hemoglobin.

Discussion

We have demonstrated *in vitro* differentiation of human ES cells to multiple hematopoietic lineages. Although *in vitro* colony assays are commonly used to study human hematopoiesis (30, 31), one concern about using colony assays to identify ES cell-derived hematopoietic cells is that the colonies could consist

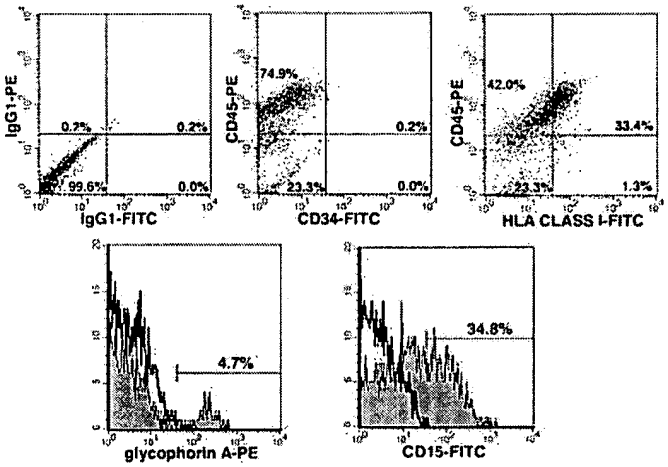

Fig. 4. Flow cytometric analysis of hematopoietic cells derived from methylcellulose colony assay of H1/S17 cells. Cells were washed free of methylcellulose before incubation with indicated antibodies and analyzed by two-color (*Upper*) or one-color (*Lower*) flow cytometry. (*Upper*) Isotype controls are shown (*Left*), and percent positive cells in each quadrant is indicated. (*Lower*) Isotype control is demonstrated by line, and indicated antibody is demonstrated by filled plot. Percent positive cells are shown by labeled marker.

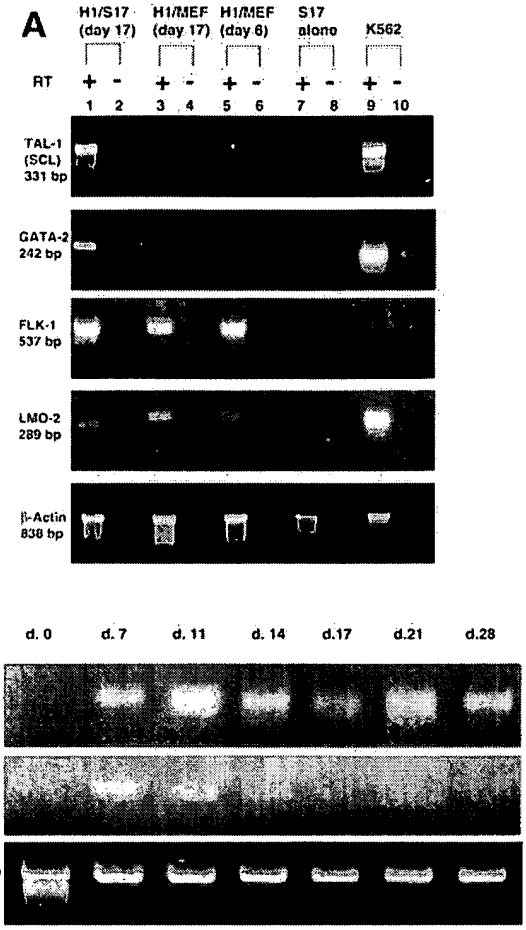

Fig. 5. Hematopoietic gene expression by RT-PCR of H1/S17 cells. (*A*) H1 cells were allowed to differentiate on either S17 cells for 17 days (lanes 1 and 2) or to differentiate on MEF cells for 17 days (lanes 3 and 4), or harvested after culture on MEFs for 6 days, before evidence of differentiation (undifferentiated H1 cells, lanes 5 and 6) and subjected to RT-PCR analysis. Irradiated S17 cells were examined to demonstrate positive bands in the H1/S17 samples were not from these feeder cells (lanes 7 and 8). The erythroleukemia cell line K562 was used as a positive control (lanes 9 and 10). Oligonucleotide primers specific for genes of interest are shown. Each sample was done with RT added (+, lanes 1, 3, 5, 7, and 9) and without RT added (−, lanes 2, 4, 6, 8, and 10) to demonstrate positive bands are not caused by genomic DNA. (*B*) Time course of hematopoietic gene expression. H1 cells were allowed to differentiate on S17 cells for the number of days indicated prior isolation of RNA for RT-PCR analysis. Day 0 (d. 0) indicates undifferentiated H1 cells. Controls of PCR done on samples without RT added did not have any positive bands (data not shown).

of nonhematopoietic cells that are able to grow in clusters that merely resemble hematopoietic colonies. However, we have used several complementary methods to demonstrate that these colonies consist of hematopoietic cells. The hemoglobin (red) of the burst-forming unit-erythroid colonies provide a distinct marker of terminally differentiating erythroid cells. Moreover, these cells are glycophorin A^+ and express normal adult globin genes as detected by RT-PCR. The esterase-positive granules in the CFU-GM-derived colonies are characteristic of granulocytes and macrophages. Additionally, the cells within the myeloid-derived (CFU-GM and CFU-M) colonies are $CD45^+$ and $CD15^+$. The CFU-Mk-derived cells (megakaryocytes) are $CD41^+$. Sorting the H1/S17 cells into $CD34^+$ and $CD34^-$ populations demonstrates enrichment of CFCs within the $CD34^+$ population, as expected for hematopoietic precursors. Although the frequency of CFCs is relatively low under the conditions of differentiation described, the yield using CD34-enriched populations is close to the number of CFCs derived from human bone marrow samples (≈100–1,000 CFCs per 10^5 bone marrow cell). Further studies to evaluate methods to derive more highly purified populations of HSCs and CFCs from human ES cells will be of interest.

The hematopoietic differentiation of human ES cells has important therapeutic implications, including the derivation of erythrocytes and platelets for transfusions, and the derivation of HSCs for hematopoietic cell transplantation. Because ES cells can be expanded without apparent limit (16), ES cell-derived blood products could be created in virtually unlimited amounts. These cells could be screened for pathogenic organisms and even potentially be genetically engineered to treat specific patients or to combat specific diseases. ES cell-derived HSCs could dramatically increase both the availability and the effectiveness of HSC transplantation for the treatment of hematologic malignancies. Recent work in mice suggests that highly purified HSCs can provide long-term engraftment across complete allogeneic barriers (32, 33). However, the dose of purified HSCs required to obtain engraftment across these allogeneic MHC barriers is high. By using human ES cells as the starting cell population, a sufficiently large dose of pure HSCs could be generated, which would permit allogeneic engraftment. Importantly, the *in vitro* derivation of HSCs capable of long-term, multilineage engraftment from mouse ES cells has so far proven an elusive goal. Mouse ES cells, however, clearly have this potential, as they routinely contribute to the definitive hematopoietic system *in vivo* when formed into chimeras with preimplantation embryos (34). Thus, the failure of hematopoietic cells derived *in vitro* from mouse ES cells to support long-term, multilineage engraftment reflects our current ignorance of hematopoietic differentiation, but does not reflect a defect in the developmental potential of ES cells.

The derivation of engraftable HSCs from human ES cells will have implications for human medicine far beyond the treatment of hematologic malignancies, as these HSCs may provide a powerful method to prevent immune rejection of other ES cell-derived tissues (35). Use of hematopoietic cell transplantation as a means to create tolerance to a solid organ transplant has been studied since the 1950s (36). Recent studies have shown

that transplantation of highly purified mouse HSCs across allogeneic barriers creates tolerance to other tissues that share the same genetic background as the HSCs (37). Other studies in both primates and humans demonstrate that hematopoietic chimerism can create a state of tolerance that permits long-term survival of transplanted organs without continued immunosuppression (38, 39). If human ES cell-derived HSCs can be used to create hematopoietic chimerism in a patient, that patient should be tolerant to other tissues derived from the same ES cells and would not require any continuous immunosuppressive treatment.

The clinical promise of human ES cell-based therapies is great; however, because these therapies will be entirely novel, serious concerns about safety and efficacy will need to be addressed before human clinical trials can be initiated. The malignant transformation of cells that have been cultured for extended periods is a particular concern. Because we also have isolated ES cells from rhesus monkeys (40), it will be possible to use these primate cells as an accurate, preclinical transplantation model for human ES cell-based therapies. Recently, the hematopoietic potential of the rhesus monkey ES cells has been demonstrated (41). These animal models, as well as continued study of methods to promote lineage specific differentiation, will facilitate the potential clinical applications of human ES cell-based therapies.

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